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14. ABSTRACT
The role of SIRT1 in breast cancer stem cell has been studied in breast cancer cell lines, human breast cancer samples, and xenograft mouse model. In breast cancer cell lines, SIRT1 inhibitors cambinol and Ex527 significantly decreased CD44 expression in MDA-MB-231, CD24 expression in MDA-MB-468, and ALDH1a positive cells in MDA-MB-231. SIRT1 inhibitors significantly blocked the cell invasion in vitro, and also inhibited cancer cell migration. SIRT1 inhibitors blocked Wnt pathway showing significantly downregulation of cyclin D1 and c-Myc. SIRT1 inhibitors significantly blocked TGFβ1 induced EMT in cancer cells. Immunohistochemitry performed on human breast cancers (N=32) showed significant high SIRT1 expression in grade 3 cancers, positive correlation between SIRT1 and vimentin, and grade 3 cancers with high stem cell expression (ALDH1a/CD44). SIRT1 expression was significantly associated with DVL3 protein. In xenograft mouse model, intramammary fat pad tumor cell inoculation generated a very good model for studying nodal metastasis for breast cancer. SIRT1 inhibitor cambinol significantly inhibited tumor growth and blocked tumor metastasis. Cancer cells became resistant to Cisplatin therapy after 2 weeks, and adding SIRT1 inhibitor cambinol blocked the cancer cell resistance. Xenogaft tumor tissue had significant lower stem cell gene expression and vimentin protein in SIRT1 treated group.

15. SUBJECT TERMS
BREAST CANCER, STEM CELL, SIRT1, SIRT1 INHIBITOR, XENOGRAFT MODEL, EMT, Wnt PATHWAY, DVL3

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INTRODUCTION

The presence of circulating tumor cells (CTCs) have been associated with poor prognosis and decreased overall survival in both operable and advanced breast cancer. The persistence of CTCs in breast cancer patients after chemoradiation therapy might be associated with stem cell-like tumor cells as the active source of cancer metastasis and remote cancer relapse, and molecular characterization of CTCs might contribute to the identification of metastatic stem cells. Many studies have shown that subsets of CTCs have stem cell phenotype expressing CD44, ALDH1 and some epithelial mesenchymal transition (EMT) markers. EMT represents the series of events converting adherent epithelial cells into individual migratory cells able to invade the extracellular matrix, and EMT plays a crucial role in cancer cell invasion and the distal metastasis in epithelial cancers. The subject of this current study is to evaluate the role of SIRT1 in breast cancer stem cells and EMT. The purpose of this study includes studying the signal transduction pathway of SIRT1 in breast cancer stem cells and EMT, exploring the therapeutic role of SIRT1 inhibitors to achieve the complete cure of breast cancer, and collecting preclinical data of SIRT1 inhibitors for future clinical trial. The scope of this study include immunohostichemitry evaluation of SIRT1 expression in human breast cancer samples, in vitro cell study for Wnt pathway and other stem cell pathways, and in vivo xenograft study to explore the effects of SIRT1 inhibitor on tumor growth and metastasis.

KEYWORDS:

Cancer stem cells, circulating tumor cells, epithelial mesenchymal transition, Wnt pathway, SIRT1, inhibitor, breast cancer, invasion, migration, xenograft, immunohistochemistry, near-infrared fluorescent (NIRF) dye, tissue growth factor, cambinol, EX527, cancer cell line, lymphovascular invasion, flow cytometry, qRT-PCR

OVERALL PROJECT SUMMARY

Task 1. SIRT1 inhibitors can induce differentiation of CSCs in breast cancer cell lines.

The task has been performed and completed. In order to study the breast cancer stem cell population in cancer cell lines, flow cytometry analysis for the most commonly used stem cell markers CD44/CD24 expression and ALDH1a activity had been used, and mammosphere assay also was used. Breast cancer cell lines (MDA-MB-231, MDA-MB-478 and T-47D) were treated with SIRT1 inhibitor cambinol and EX527.

Flow cytometry analysis for CD44/CD24 expression showed a significant reduced expression of CD44 expression in the triple negative breast cancer cell line MDA-MB-231 and MDA-MB-468. MDA-MB-231 cancer cells were negative for CD24 and positive for CD44, supporting the high grade nature of the tumor cells. When the cells were treated with SIRT1 inhibitors cambinol (25 μM) and Ex527 (50 μM) for 24 hours, the cells showed significantly decreasing CD44 expression with a marked left shift (Figure 1A and 1B). Another triple negative breast cancer MDA-MB-468 cells showed coexpression of both CD24 and CD44, which was different from MDA-MB-231. Studies had shown that CD24 expression in MDA-MB-468 cells was important for its invasive ability. With SIRT1 inhibitors cambinol (25 μM) and Ex527 (50 μM) treatment, MDA-MB-468 cells showed significant loss of CD24 expression and only slight loss of CD44 (Figure 2A and 2B). The none triple negative, high grade breast cancer T-47D cells had no significant CD44 expression. All studies had been repeated for at least 2 times and showed similar results.
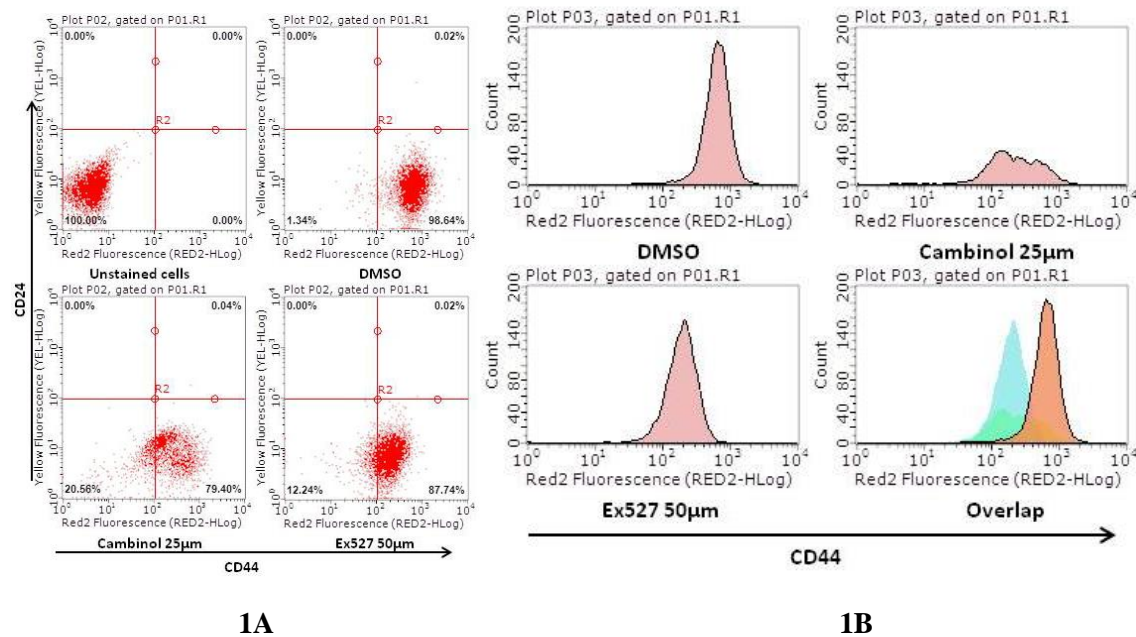
1A

1B

Figure 1. Figure 1A shows the dot plot of flow cytometry analysis for CD24/CD44 expression in MDA-MB-231 cancer cells, and 1B shows the histograph and the overlap of the CD44 intensity in SIRT1 inhibitor treated cells comparing to DMSO control (orange-control; green-cambinol treated; blue-Ex527 treated).
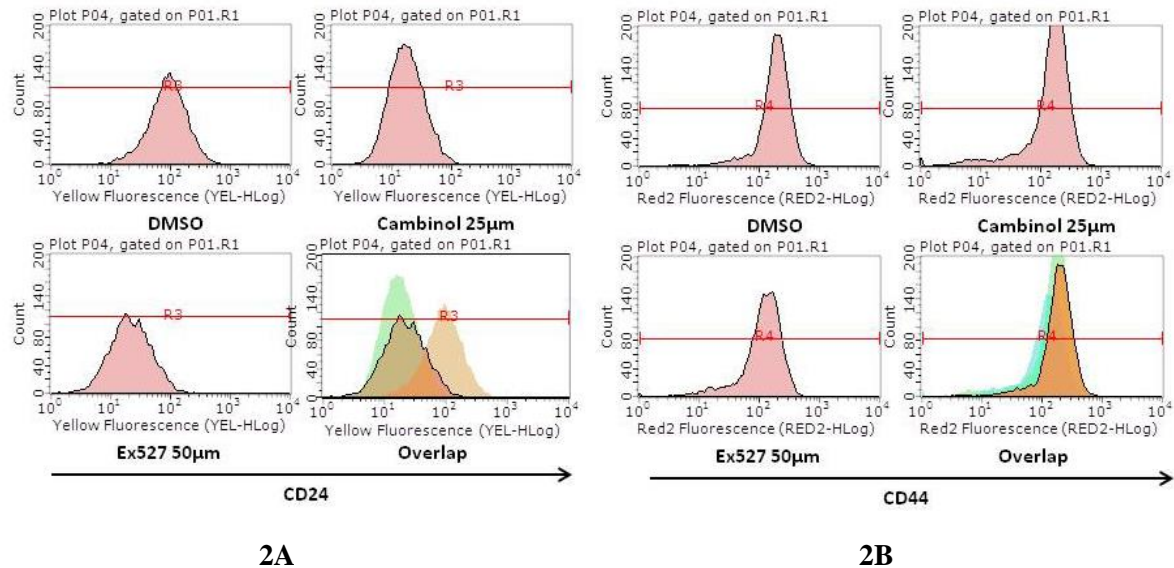

2A

2B

Figure 2. The histograph and the overlap of the CD24 intensity (2A) and the CD44 intensity (2B) in SIRT1 inhibitor treated cells comparing to DMSO control (orange-control; green-cambinol treated; blue-Ex527 treated).

Adeflour for ALDH1a was performed on MDA-MB-231 cells. SIRT1 inhibitor cambinol treated cells showed dramatic decreased ALDH1a positive population, from 45% in the DMSO control to 6.9% in the cambinol 25 μM treated cells (Figure 3). T-47D cells had only minimal ALDH1a positive cells. Mammosphere assay was performed according the manufactory instruction. Triple negative breast cancer MDA-MB-231 and MDA-MB-468 did not form tight and large spheres, and T-47D cells were used for

the assay. The mammosphere assay showed a significant reduce of sphere formation in T-47D when treated cells with SIRT1 inhibitor cambinol and Ex527.
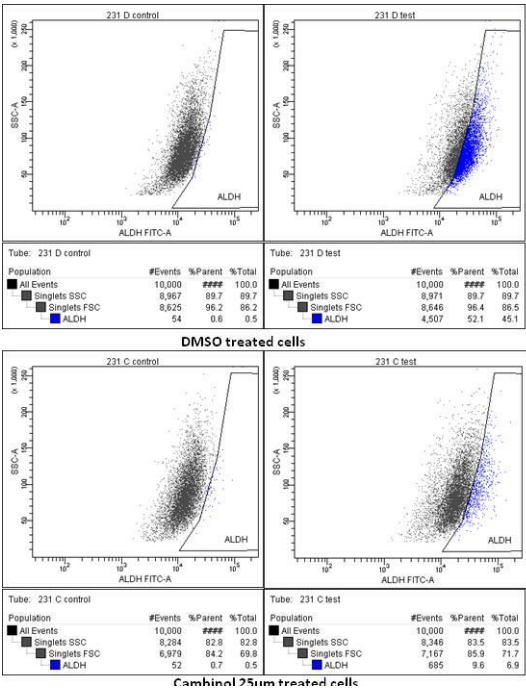

Figure 3. Breast cancer MDA-MB-231 cells were treated with DESO and SIRT1 inhibitor cambinol 25 μM for 24 hours. Cells were harvested and prepared according to the Adeflour manufactory instruction for the assay. For each sample, a half of the sample was inactived to serve as the sample negative control, and the ALDH1a positive cells were analyzed with flow cytometry. The DMSO treated MDA-MB-231 cells had 45% of ALDH1a positive cells, and the cambinol treated cells had only 6.9% ALDH1a positive cells. The study had been repeated for 3 times with the similar findings.

On the next step, cancer stem cell gene expression profile with SIRT1 inhibitor treatment using qRT-PCR was performed on several cell lines, and stem cell genes including Nanog, SOX-2 and Pou5F1 were analyzed. After cambinol treatment for 48 hours, MDA-MB-231 cells showed significantly gene expression down regulation for all three genes (4A). T-47D cells showed similar findings (4B)
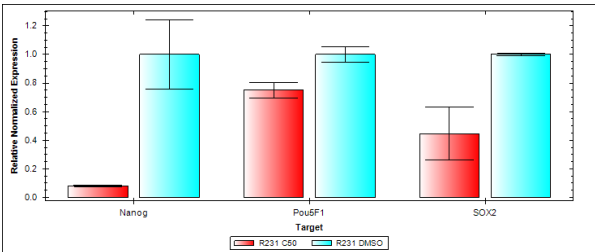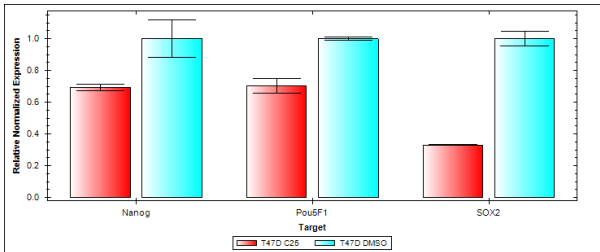

4A   **4B**

T-47D cancer cells showed very good response to TGFβ1 stimulation compared to triple negative MDA-MB-231 cells, so T-47D was used to study for TGF β1 study. The western blot showed SIRT1 inhibitors cambinol and Ex527 also reduced the protein levels of Nanog and Sox-2 in T47D cells, corresponding to the qRT-PCR results. Furthermore, specifically knock down SIRT1 expression with SIRT1 small inhibitor RNA significantly reduced SOX-2 protein and slightly decreased Nanog protein in T-47D cells (5A). Since cancer cells with stem cell characters share some similar features with cancer cells undergoing EMT, we studied some molecular markers of EMT on the cancer cell lines. For cancer cells undergoing EMT, they loss E-cadherin and gain expression of N-cadherin, vimentin and smooth muscle actin. T-47D cells were stimulated with 1 ng/ml TGFβ1 with DMSO or cambinol 25 μM, and the gene expression was compared after 24 hours. SIRT1 inhibitor cambinol significantly blocked TGFβ1 induced vimentin, N-cadherin and SMA expression (5B).

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In summary, *in vitro* study in several breast cancer cell lines using flow cytometry analysis for CD24/CD44 expression and Adeflour for ALDH1a positive cells, functional study with mammosphere assay, and qRT-PCR for gene expression profile showed SIRT1 inhibitors can induce cancer cell differentiation by reducing cancer stem cells and blocking TGFβ1 induced EMT. For Task 1, all proposed studies were completed, and some additional experiments such as mammosphere functional assay and gene expression profile with qRT-PCR had been added.
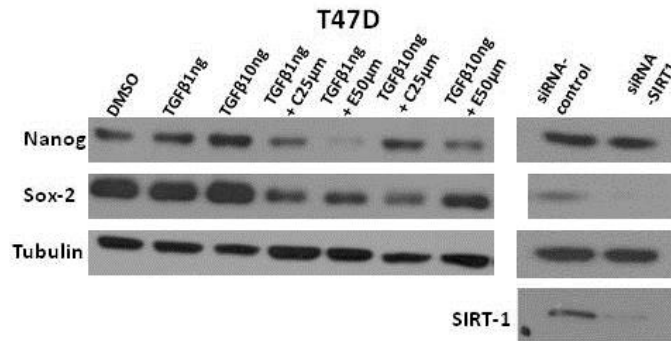

5A
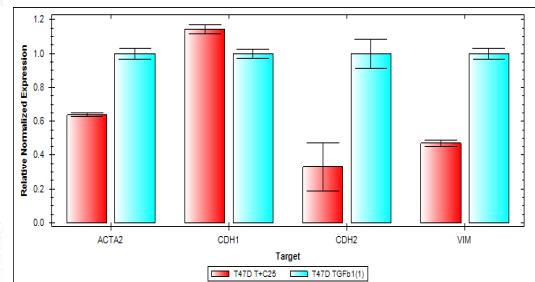
5B

Task 2. Human breast cancer cells (from patient's samples) with CSC features have SIRT1.

32 breast cancers with variable grades and stages had been selected after the IRB approval, and SIRT1, several CSC markers (CD44, ALDH1a, SOX-2, OCT-4, Nanog and CD133) and EMT markers (vimentin, E-cadherin, Snail and Twist) had been used for immunohistochemistry. CD44/ALDH1a double staining has been used. All markers were blindly scored using H score formula, with staining intensity (0-3) times the cell percentage (0-100). The scores ranged from 0-300. Chi square and Pearson correlation coefficient were used for data analysis. The immnohistochemical results showed significant correlation between SIRT1 expression and breast cancer tumor grades, and grade 3 breast cancers had significantly high SIRT1 expression compared to grade 1/2 breast cancers (6A). Pearson correlation coefficient analysis showed significant positive correlation between SIRT1 and vimentin (p=0.0001) (6B). High grade breast cancers showed decreased E-cadherin, but there was no statistic significance.
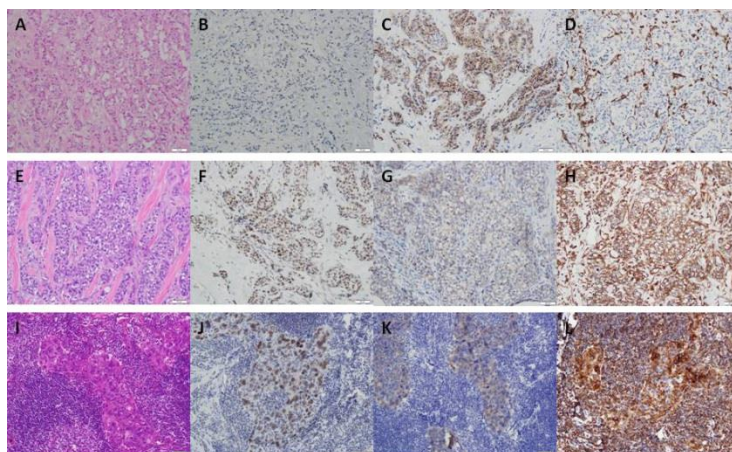

6A
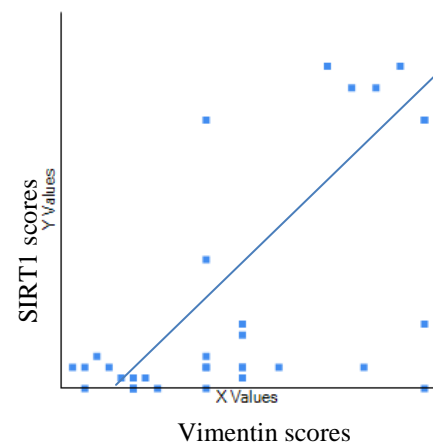
6B

However, the correlations between the individual CSC markers showed no significant correlation, and no individual stem cell marker showed correlation with tumor grades. All 32

tested breast cancers were negative for OCT-4, and Nanog showed some cytoplasmic staining in some cases but no nuclear positivity. A different approach to evaluate the CSC in breast cancer samples with co-localization of 2-3 CSC markers showed that ALDH1a/CD44 coexpression/co-localization was significantly associated with high grade breast cancers (56% G3 vs 7% G1/2, p=0.007), and ALDH1a co-localization with one or more other markers (CD44, CD133 or SOX-2) also observed more in high grade cancers (72% G3 vs 21% G1/2, p=0.01). So, double staining with ALDH1a/CD44 will be performed in the tumor tissue to confirm the current finding.

In summary, high grade breast cancers showed significant high SIRT1 expression, high vimentin expression, high percentage tumor cells positive for one or more cancer stem cells (especially double positive for ALDH1a/CD44). Double staining with cocktails antibodies of ALDH1a/CD44 will be performed to better validate the current finding.

Task 3. SIRT1 inhibition can decrease metastasis, induce differentiation of CSCs, reduce EMT, and increase tumor cell sensitivity to chemotherapy in xenograft mouse model.

The animal study protocol was approved by both UT and DOD animal committee, the *in vivo* study was performed and whole body image with both PET and iRFP techniques by collaboration with Dr. Eva Sevick at the image core center, Institution of Molecular Medicine at UTHSC at Houston. A mammary fat pad cell injection was used to mimic the human breast cancer and hoped to reach the lymph node metastasis. The first group 35 nude mice were used for MDA-MB-231 cell inoculation. The second group mice have not been purchased for T-47D cell inoculation. MDA-MB-231 cells were first tested because it had been shown to have high metastatic potential than other breast cancer lines. The image facility was changed from MD Anderson Cancer Center to the image core center at UTHSC because the difficulty of transferring animals between 2 different institutions.

7A   7B
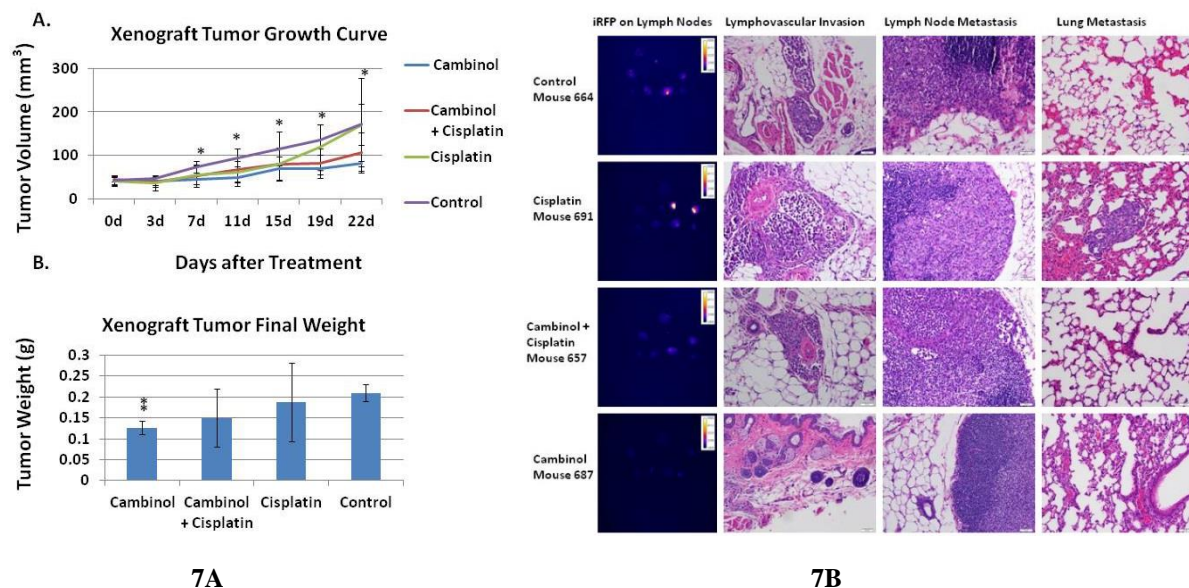
The *in vivo* study with MDA-MB-231 cells showed some very good and exciting results. SIRT1 inhibitor cambinol treated mice (N=3) had significant slow tumor growth (indicated in the growth curve), and tumor volume showed minimal changes during the entire 3 weeks (7A). The DMSO control mice (N=5) had tumor volume tripled during the same period. Cambinol+cisplatin group (N=4) had similar tumor growth curve as the cambinol group with slightly large tumors. The cisplatin treated group (N=5) showed similar slow tumor growth curve at the first 2 week, but tumor started to grow very fast at the

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beginning of the 3rd week and reached to a similar tumor volume as the DMSO control group at the end. The growth curve indicated a sudden loss cisplatin response in tumor cells. When SIRT1 inhibitor cambinol was used together with cisplatin, the tumor fast growing phase did not occur and tumor remained in a very slow growth curve compared to the control group. **This result suggested that SIRT inhibitor cambinol could block the resistance of MDA-MB-231 cells to cisplatin.** The final tumor weight showed significant small tumors in the SIRT1 inhibitor cambinol treated mice. The intramammary fat pad inoculation of MDA-MB-231 cells generated a very good model to study the lymph node metastasis. Our study showed significant lymph node metastasis than blood stream metastasis (only one lung metastasis). Using iRFP whole body image and lymph node image study, positive lymph node metastasis was found in 5/5 control mice and 15/37 nodes, 4/5 cisplatin treated mice and 9/40 nodes, 3/4 cambinol+cisplatin treated mice and 5/40 nodes, and 0/3 cambinol treated mice and 0/20 nodes. H&E sections of skin showed marked lymphovacular invasion in the control mice but not in the cambinol treated mice (7B).
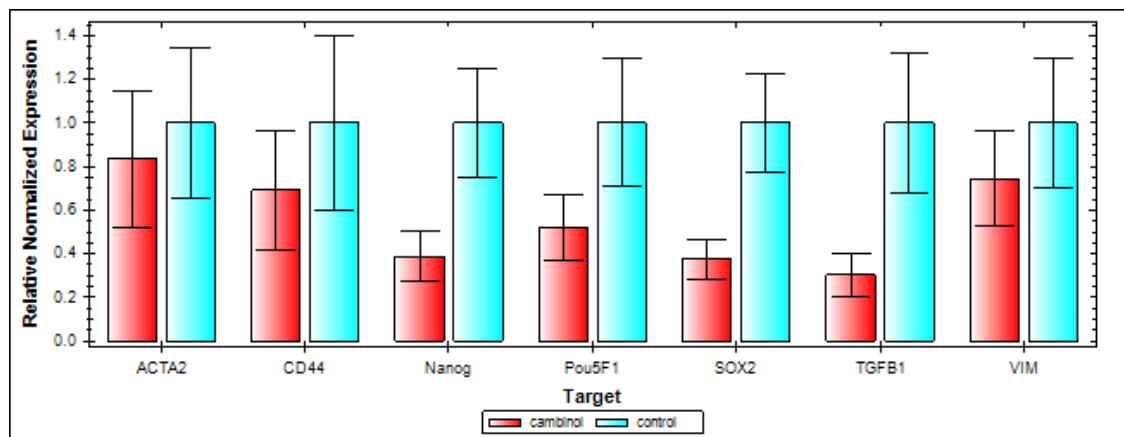

8A
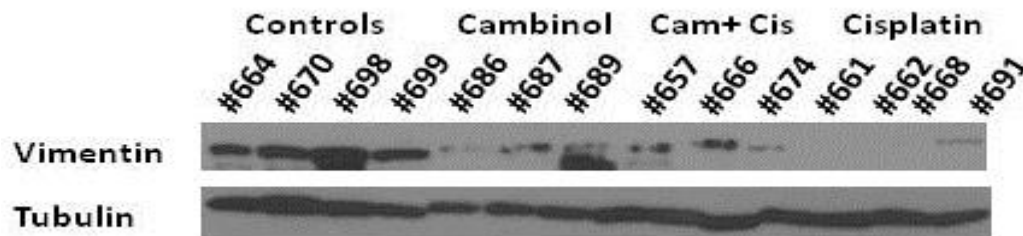
8B

qRT-PCR gene expression study showed significant down regulation of cancer stem cell genes (Cd44, Nanog, Pou5F1 and SOX-2) and EMT genes (TGFβ1, vimentin and SMA) in cambinol treated mice compared to DMSO control mice (N=2) (8A). Surprisingly, western blot showed low vimentin protein levels in all treated groups compared with DMSO controls (8B). The result indicated that vimentin may not be the good marker for tumor metastatic potential.
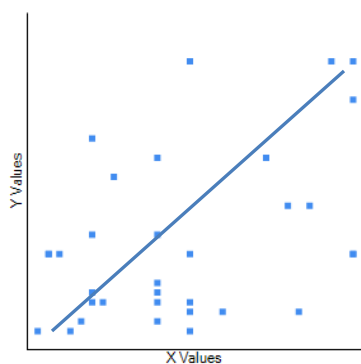
SIRT1 inhibitors block breast cancer cell invasion was also confirmed in in vitro invasion assay (Figure 9). Three triple negative breast cancer lines (MDA-MB-231, MDA-MB-468 and BT-549) were treated with cambinol 25 μM with DMSO as control. After 48 hours, the invasive cells were stained with Diff-Quik and counted. Cambinol treatment significantly blocked all 3 cancer cell invasion.

A | B | C — DMSO

D | E | F — C25 µM

MDA-MB-231 | MDA-MB-468 | BT-549
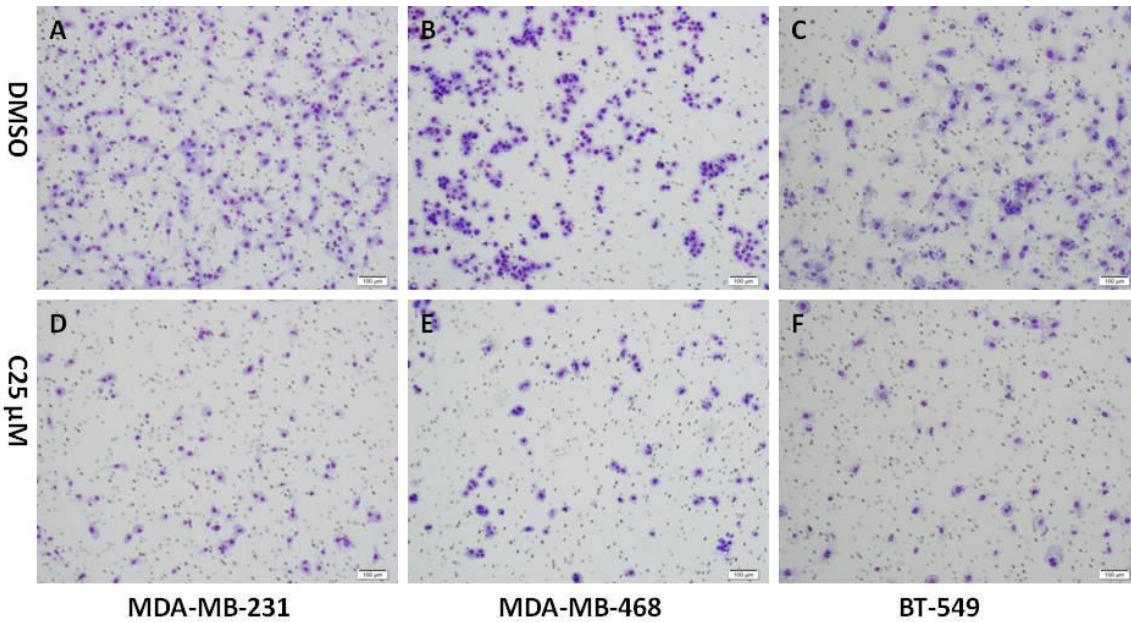
In summary, xenograft model with nude mice and MDA-MB-231 cells demonstrated that SIRT1 inhibitor cambinol decreased breast cancer growth, blocked cancer metastasis *in vivo* and invasion *in vitro*, and possible rescued cancer cells from resistance to cisplatin. Gene expression profile indicated SIRT1 inhibitor cambinol down regulated cancer stem cells *in vivo*.

Task 4. Wnt pathway is highly activated in breast CSCs and EMT of human breast cancer specimens.

Immunohistochemistry for SIRT1, Dvl3, beta-catenin and activated beta-catenin has been performed on the 32 cases of breast cancers, and SIRT1 expression was positive significantly correlated with Dvl 3 protein expression in breast cancers (9A). Just like the findings in literature, we did not see beta-catenin nuclear translocation in breast cancer samples, and there was no correlation between SIRT1 and activated beta-catenin.


9A
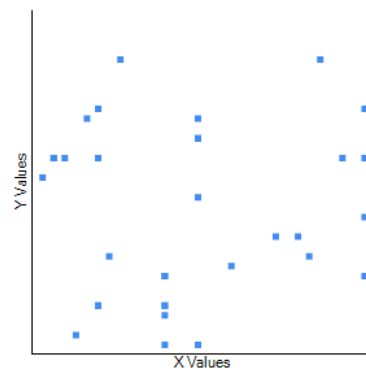
9B

In breast cancer cell lines, SIRT1 inhibition was clearly associated with inactivation of Wnt pathway by significantly down regulation of Wnt pathway downstream molecules such as cyclin D1, c-Myc and c-Jun (10A). Another evidence that SIRT1 inhibitor blocks Wnt pathway

is SIRT1 inhibitor cambinol and Ex527 significantly decreased DVL3 protein in several breast cancer lines (10B).
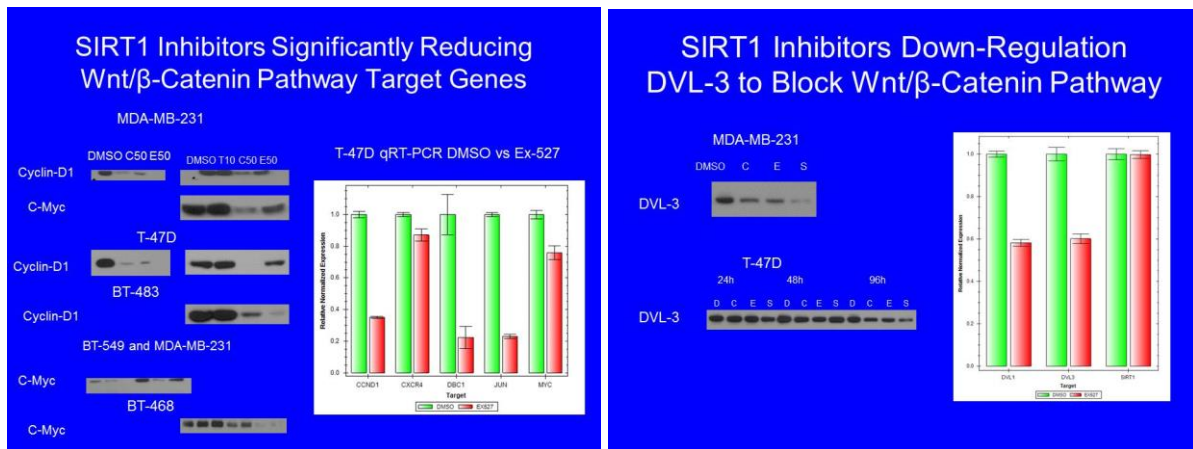
10A   **10B**

The downstream targets of Wnt pathway in breast cancer samples such as GSK, PKC, CK1, c-Myc and cyclin D1, have not been studied yet, and the task remains uncompleted.

In summary, the immunohistochemistry performed on human breast cancers showed SIRT1 was significantly associated with DVL3 expression, and breast cancer cell line study showed SIRT1 inhibitors significantly blocked Wnt pathway showing the reduction of c-Myc, cyclin D1 and c-Jun. SIRT1 inhibitors are likely regulated Wnt pathway by regulation of DVL3 protein.

Task 5 . Wnt pathway is blocked in the SIRT1 inhibition xenograft tumor tissue, which is responsible for inducing differentiation of CSCs and reducing EMT.

Immunohistochemsitry for SIRT1, vimentin and E-cadherin has been performed on the tumor tissue from xenograft mice, and western blot was also performed. Cambinol treated tumors showed reduced vimentin expression, supporting SIRT1 inhibitor blocks EMT. The Wnt pathway in the xenograft tumors has not been performed.

In summary, western blot for Wnt downstream molecules and DVL3 will be performed on the xenograt tumor tissue.

Task 6. Using cell line *in vitro* study to demonstrate that SIRT1 regulates CSCs and EMT through activation of Wnt pathway via interaction with Dvl proteins.

Breast cancer cell lines MDA-MB-231 and T-47D had been used *in vitro* study, and SIRT1 inhibitors (cambinol and Ex527) and SIRT1 siRNA were used to study the Wnt pathway. SIRT1 inhibitors and siRNA significantly blocked Wnt pathway showing down regulation of cyclin D1, c-Myc and c-Jun, and reduced beta-catenin protein levels, and reduced Dvl-3 protein levels. qRT-PCR study showed SIRT1 inhibitors and siRNA significantly down regulated cyclin D1, c-Myc and c-Jun gene expression, and down regulated several stem cell markers including SOX-2 and Oct-4. We also explored the possibility of the role of SIRT1 regulation of TGFβ1 pathway in the EMT signal transduction, and we found that SIRT1 clearly regulated TGFβ1 activity (Figure 11).
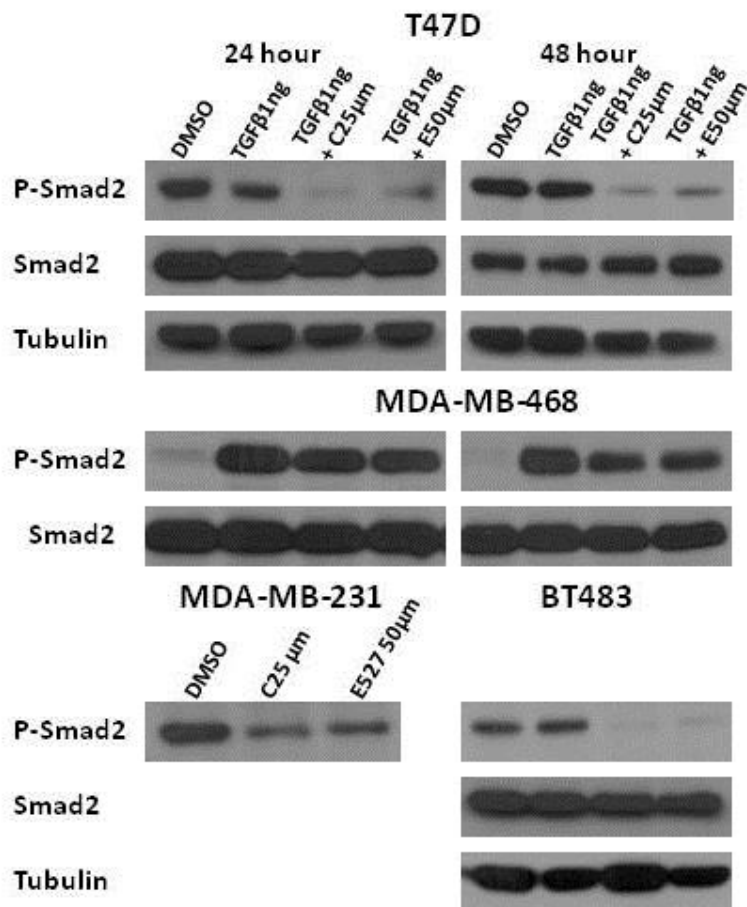
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Figure 11.

In summary, SIRT1 inhibitors regulate breast cancer stem cells by down regulation of Wnt pathway and blocking TGFβ1 pathway.

KEY RESEARCH ACCOMPLISHMENTS:

- SIRT1 inhibitors reduce breast cancer stem cell population
- SIRT1 inhibitors block breast cancer EMT
- SIRT1 inhibitors can prevent cancer resistance to chemotherapy drugs
- ALDH1a/CD44 double staining/co-localization serves better marker for breast cancer stem cell in human breast tissue
- SIRT1 inhibitors block breast cancer invasion and migration *in vitro* and block cancer metastasis in xenograft model
- SIRT1 regulates Wnt pathway likely through affecting DVL3 protein
- SIRT1 regulates TGFβ1 pathway
- Intramammary fat pad inoculation is better xenograft model for studying nodal metastasis in breast cancer

CONCLUSIONS:

The role of SIRT1 in tumorigenesis remains controversial, i.e. whether it acts as a tumor promoter or a tumor suppressor, and the role of SIRT1 in breast cancer stem cells has never been explored. SIRT1 is strongly expressed in embryonic stem cells, and SIRT1 downregulation is necessary to establish correct

and specific differentiation programs. Cancer stem cells may serve as cancer reservoir for breast cancer recurrence and distant metastasis. One significant challenge of cancer stem cell study is to identify possible stem cells with specific markers. SIRT1 induces EMT with epigenetic silencing of E-cadherin expression (the hallmark of EMT) in prostate cancer and enhances cancer migration and metastasis; similar findings are observed in mammary epithelial cells. The current study has been designed to study the import and critical issues about finding the cure for breast cancer-targeting to the cancer stem cells.

The significant findings of the current study prove that SIRT1 inhibitor can be used as a therapeutic target of breast cancer, especially in high grade breast cancer, such as triple negative breast cancer. Small molecular weights of SIRT1 inhibitors also provide potential therapeutic management on breast cancer patients with brain metastasis, and SIRT1 inhibitors can be given locally. SIRT1 inhibitors can significantly reduce the cancer stem cells, and it may provide a final cure on breast cancer.

The future study includes subclincal trial using SIRT1 inhibitors on primary tumor tissue and primary tumor xenograft model, using SIRT1 inhibitors in CSF metastatic mouse model, and using local applying of SIRT1 inhibitors in tumor mass. Several proposals had been submitted for additional supports.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Abstracts:

1. Songlin Zhang, Min Li, Baoxiang Guan, Robert Brown. SIRT1 inhibitors significantly reduce cancer stem cells and block epithelial mesenchymal transition in breast cancer cells. 2014 San Antonio Breast Cancer Symposium, San Antonio.
2. Frances Compton, Min Li, Songlin Zhang. High SIRT1 expression associated with epithelial mesenchymal transition in breast cancer. 2015 ASCP Annual Scientific Meeting, Long Beach.
3. Frances Compton, Min Li, Songlin Zhang. Cancer stem cell marker expression associated with high grade breast cancer. 2015 CAP Annual Meeting, Chicago.

Presentation:

1. SIRT1 as a therapeutic target in breast cancer. Department Research Seminar, UTHSC, Houston 03/21/2014
2. SIRT1 as a therapeutic target in breast cancer. Grand Round, medical oncology, UTHSC, 09/15/2014

INVENTATIONS, PATENTS AND LICENSES:

Nothing to report.

REPORTABLE OUTCOMES:

Nothing to report.

OTHER ACHIEVEMENTS:

Additional grant proposals had been submitted to another DOD grant and one Texas cancer research grant as PI, and several other grants as co-PI or collaborator.

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APPENDICES:

None.